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Final Report on LANL Subcontract No. 9-XEO-M6960-1

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I. ABSTRACT

This subcontract was undertaken by Dr. Richard A. Palmer of the Department of Chemistry, Duke University, to develop (in collaboration with Dr. Brian Dyer and Dr. W.H. Woodruff of CLS-4 and INC-4, LANL, respectively) an experiment in time-resolved step-scan FT-IR for use in dynamic photophysical studies of cytochrome-C oxidase and other heme proteins. To this end, an instrument was assembled which incorporated a step-scan FT-IR spectrometer developed at Duke and several components on loan from LANL, including particularly a frequency doubled Nd-YAG pump laser and a pair of gated integrators. The specific objective was to obtain information on the slower steps (μ s-ms) of the reassociation of CO with the heme iron of cytochrome-C oxidase and myoglobin after its photodissociation by a 10 ns pulse from the Nd-YAG laser and to correlate the CO dynamics with associated conformational dynamics in the protein, as indicated by changes in the CO and amide vibrational bands. The step-scan FT-IR probe allows time-resolved spectra to be obtained over the entire mid-IR region ($400\text{-}4000\text{ cm}^{-1}$). Although the D_2O solvent occludes much of this region, observation in the CO stretching and Amide I, II, and III mode regions is still possible in the D_2O windows. Initial results were obtained on cytochrome-C oxidase, but, because of the sample requirements, the more readily available carbonmonoxymyoglobin (MbCO) was chosen for study during the rest of the technique development. The initial results on myoglobin are encouraging as to the original objective of simultaneously monitoring both the reassociation of the CO and the protein conformational changes, but comparison of the FT-IR results with those obtained from the same sample at LANL by use of a tunable IR diode laser show significant differences. There is strong indication that the total laser power required to get a useful dynamic difference signal in the FT-IR experiment is causing a significant temperature-induced reversible conformational change in the protein which is much larger than that associated with the dissociation/reassociation of the CO. However, static difference spectra recently obtained at Duke and at LANL call into question the thermal change hypothesis.

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II. TECHNIQUE DEVELOPMENT

The development of a reliable time-resolved step-scan FT-IR technique for use in photophysical studies of heme proteins was the initial goal of the project. The advantages of FT-IR for such studies are those of high throughput, wide spectral range, and relatively high resolution. The step-scan mode of FT-IR in principle allows greater flexibility in the application of dynamic techniques to FT-IR than does the standard continuous-scan method.

The plan was to use a powerful Nd-YAG laser at Duke as the pump in these experiments. Dr. Mark Schauer was employed using subcontract funds to convert the laser from its industrial configuration to research use. Although this conversion went as planned, it was later discovered that the pulse structure and peak power of this laser were not suitable for the experiment. Therefore, it could not be used and was replaced by the loan of a Nd-YAG laser and doubling optics from CLS-4. This laser was already known to have the correct range of pulse characteristics for the experiment. However, it was seriously damaged during shipment from LANL and required considerable time on the part of Dr. Schauer to be put back in operation. This involved replacing all the flashlamps and, ultimately, the laser head board as well.

These instrumental problems, along with others involving the boxcar integrators on loan from LANL, delayed the initial experiments until the spring of 1991. At this time several experimental strategies for adapting the experiment to the step-scan FT-IR instrument were tried and eventually the current configuration and method was determined to be the most effective. Although initial results were obtained for cytochrome-C oxidase (Cco) during this time, it was decided to switch samples to carbonmonoxy myoglobin (MbCO) in order to complete the instrumental development phase since it was a less labor-intensive sample to prepare.

In accomplishing the goal of technique development an apparatus was eventually assembled consisting of:

1. IBM/Nicolet IR-44 bench-top FT-IR spectrometer, previously converted in our labs to optional step-scan operation.
2. Quanta Ray DCR 1A mode-locked, Q-switched Nd-YAG laser with Harmonic Generator Model I, capable of second, third, and fourth harmonic operation.
3. Wavetek Model 130 Function Generator.
4. Stanford Research Systems, Inc. Four Channel Digital Delay/Pulse Generator Model DG 535.
5. Stanford Research Systems, Inc. Gated Integrator Modules (2), Model 250.

The configuration of the instrument is illustrated in Figure 1. The schematic of the step-scan FT-IR spectrometer and its specifications are given in Figure 2, along with an illustration of the principle of its method of phase modulation mirror control and stepping. It is important to note that the phase modulation is intrinsic to the mirror control, and is also used to provide the

infrared modulation for the AC-coupled mercury-cadmium-telluride (MCT) detector by adjusting its amplitude to $2\lambda_{\text{HeNe}}$ for maximum modulation efficiency at 2300 cm^{-1} .

In the collection of time-resolved photophysical data the function generator is the master oscillator (typically 400 Hz) and synchronizes the interferometer mirror phase modulation and the external trigger for the pulse generator. The pulse generator then sets a 10 Hz repetition rate for the laser, and sequentially triggers the flashlamp, Q-switch and gated integrator modules. The gated integrator takes two samples (typically a gate width of $15\text{ }\mu\text{s}$), one channel before and one at a delay time t after the laser pulse. By spacing the gates exactly 2.5 ms apart, both samples are taken at the same phase of the (phase) modulation. This is necessary since otherwise the small transient signal would be completely lost in the presence of the large phase modulation. The spacing of the gates and the laser flash with respect to the phase modulation is illustrated in Figure 3, and the more complete timing diagram is shown in Figure 4. The AD converter samples the gated integrators through a multiplexer, over the period that the mirror is stationary (oscillating about a fixed point with a fixed frequency and amplitude). The computer averages the sample points and sorts them into two separate interferograms. The two interferograms are Fourier transformed to produce two spectra. The difference spectrum (ΔA , "after" minus "before") shows which bands have changed as a result of the laser perturbation. The integrity/feasibility of this experimental set-up has been tested and verified by several null experiments. On average three laser flashes occur during each of the sampling periods at each mirror position. The sampling occurs at intervals of $1\lambda_{\text{HeNe}}$ which gives a free spectral range of $7900\text{--}0\text{ cm}^{-1}$. The 2203 data points and consequent mirror scan length of 0.125 cm yield a resolution of 8 cm^{-1} and a data collection time of ca. 25 minutes. It has been found that the co-addition of several 25 minute scans produces a greater improvement in SNR than the acquisition of a single scan of equivalent total collection time. At present, the time resolution range of the experiments is 100 ns, although new equipment should significantly improve the spectral and temporal resolution, and in general the quality of the experimental results.

III. RESULTS

Figure 5 is a typical step-scan FT-IR absorbance spectrum of 10 mM MbCO/D₂O, with the band assignments provided. A 10 mM solution in a $25\text{ }\mu\text{m}$ pathlength cell provides the optimum absorbance (ca. 1.8 A.U.) in the Amide I band. A series of difference spectra at various delay times are shown in Figure 6. It is of interest to note the expected progression in the CO bleach. At longer delay times after the laser pulse, more CO has recombined and the difference signal becomes smaller. However, the Amide I does not appear to follow the same dynamics; its maximum bleach occurs some time later after the earliest time probed. In addition, when comparing these results to those of our collaborators at LANL, the relative intensities of the CO

and Amide I bands are reversed and structure appearing in the LANL difference spectrum in the region of the Amide I band does not appear in the FT-IR difference spectrum, despite similar resolution in the two experiments. The LANL difference spectrum is shown in Figure 7.

After considering a number of possible reasons for the contrast between the FT-IR and laser diode results, the evidence seemed to point to a thermal effect. (The pump power density in the LANL experiment is reported to be ~ 3 mJ/pulse/cm² versus 10 mJ/pulse/cm² in the FT-IR experiment, and the sample concentrations, 1 mM versus 10 mM, respectively.) It was concluded that, despite the similar pump power density in the two experiments, the greater total power in the larger diameter pump beam (1 cm versus 0.5 mm) and the higher sample concentration was resulting in a reversible thermal conformational change in the protein in the FT-IR experiment. To test this hypothesis the FT-IR experiment was repeated with a sample of deoxymyoglobin. In deoxyMb, the iron is in its reduced state (+2), but there is no ligand in the sixth coordination position. Thus, if the protein conformational change is only in response to ligand dissociation, there should be no Amide I difference spectrum since there is no ligand to photodissociate. The UV-Vis spectrum of the sample is shown in Figure 8, confirming it to be deoxyMb. The resulting time-resolved step-scan FT-IR difference spectrum is shown in Figure 9. These results support the hypothesis that the laser pulse used in the FT-IR experiment is causing thermally-induced protein motion on top of the desired ligand dissociation-induced dynamics.

In an effort to further corroborate the above results and conclusion, a static difference spectrum was measured. In this experiment, the spectrum of a sample of MbCO was subtracted from the spectrum of a sample of deoxyMb, each collected independently using conventional continuous scan FT-IR. Since in its photodissociated form MbCO should be similar in all respects to deoxyMb, this static difference spectrum should be similar to the dynamic difference spectrum, using a laser pulse to create the deoxy species. A similar FT-IR experiment was carried out at LANL. The Duke and LANL static difference spectra are shown in Figures 10 and 11, respectively. It is clear that a similar contrast persists between the static spectra as was found between the dynamic spectra. This tends to call in to question the thermal change hypothesis, and yet the deoxyMb experiment described above seems almost conclusively to prove it. Thus the origin of the difference between the two experiments remains in question.

There are several further FT-IR experiments to perform which may resolve this question. The first of these is to reduce the probe spot size and the pump laser diameter and power in order to approximately match the parameters in the LANL experiment. However, considering the magnitude of the changes observed at LANL, there is some question whether the step-scan technique (using the IR 44) will have adequate signal to noise to resolve the changes. Therefore, the experiment will probably be carried out using the recently completed Nicolet System 800 step-scan instrument, which promises to offer as much as an order of magnitude greater signal to

noise compared to the IR 44. Other more sophisticated time-resolved FT-IR instrumentation suitable for these experiments will also soon be available in our laboratories.

IV. PRESENTATIONS

R.A. Palmer, J.L. Chao, R.M. Dittmar, V.G. Gregoriou, and S.E. Plunkett, "Investigation of Time-Dependent Phenomena by Use of Step-Scan FT-IR" International Symposium on Advanced Infrared Spectroscopy, Development and Application of Time-Resolved and Two-Dimensional Infrared Spectroscopy, Tokyo, Japan, March 23-25, 1993.

R.A. Palmer, V.G. Gregoriou, S.E. Plunkett, R.M. Dittmar, and J.L. Chao, "Time-Resolved, Phase-Resolved and 2D Step-Scan FT-IR Spectroscopy" Pittsburgh Conference, Atlanta, GA, March 8-12, 1993.

S.E. Plunkett, M.W. Schauer, V.G. Gregoriou, R.A. Palmer, J.L. Chao, R.B. Dyer, and W.H. Woodruff, "Time-Resolved Step-Scan FTIR of Protein Dynamics" 44th Southeaster - 26th Middle Atlantic Combined Regional American Chemical Society Meeting, Arlington, VA, December 6-9, 1992.

S.E. Plunkett, M.W. Schauer, V.G. Gregoriou, J.L. Chao, R.A. Palmer, R.B. Dyer, P.O. Stoutland, K.A. Peterson, T. Causgrove, K.A. Bagley, and W.H. Woodruff, "Time-Resolved Vibrational Studies of Heme Proteins" Federation of Analytical Chemistry and Spectroscopy Societies (FACSS), Philadelphia, PA, September 20-25, 1992.

S.E. Plunkett, M.W. Schauer, J.L. Chao, R.A. Palmer, R.B. Dyer, and W.H. Woodruff, "Time-Resolved Step-Scan FT-IR of Protein Dynamics" Gordon Conference on Vibrational Spectroscopy, Wolfeboro, NH, August 3-7, 1992.

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Figure 1. Time-resolved step-scan FT-IR experimental set-up. The phase modulation from the function generator can be varied from 100-1000 Hz. The gate width of the two gated integrator channels is variable from 100 ns up to 50 ms, with a variable delay time of 0 ns up to 97.5 ms.

INTERFEROMETER

Michelson

Airbearing, solenoid linear motor

Viscous damping

Maximum resolution: $1/2 \text{ cm}^{-1}$

Maximum free spectral range

(step-scan): $31,600 \text{ cm}^{-1}$

Maximum retardation: 2 cm

Stepping frequency: 0 - 100 Hz

PM amplitude: $< 5 \text{ nm}$ - $> 5 \text{ }\mu\text{m}$

PM frequency: 100 - 1000 Hz

Rapid-scan optional

OPTICS

70 W global (40 mW on the sample)

f/4.2; beam diameter 3.5 mm

Ge-on-KBr or Si-on-Quartz beamsplitter

COMPUTER

IBM PC-AT, modified IR-14 software

Maximum collection rate: 22 min for 8 cm^{-1}

and a free spectral range of 7900 cm^{-1}

References

1. M.J. Smith, C.J. Manning, R.A. Palmer, and J.L. Chao, *Appl. Spectrosc.* **42**, 546 (1988).
2. R.A. Palmer, C.J. Manning, J.A. Rzepiela, J.M. Widder, and J.L. Chao, *Appl. Spectrosc.* **43**, 193 (1989).
3. C.J. Manning, J.L. Chao, and R.A. Palmer, *Rev. Sci. Instrum.*, **62**, 1219 (1991).

INTERFEROMETER SCAN CONTROL

LASER INTERFERENCE SIGNAL

Figure 2. Duke-IBM step-scan instrument. The theory of position control and instrument specifications are described.

Phase modulation: $2 \lambda_{HeNe}$ at 400 Hz

Rep. rate: 10 Hz, or every 40 PM cycles.

Figure 3. The temporal relation of flashlamp and laser firing and gate sampling to the 400 Hz phase modulation. The two gates are of variable width and spaced 2.5 ms apart. The second gate opens at a variable delay time t , after the laser pulse. Since the phase modulation is 400 Hz (2.5 ms), 40 PM cycles occur within each 10 Hz (100 ms) repetition rate. T_0 indicates a new 10 Hz cycle.

NOT TO SCALE

Figure 4. The timing diagram showing the temporal relation between the various components.

Figure 5. Absorbance step-scan FT-IR spectrum of 10 mM Horse MbCO/D₂O, 25 μ m pathlength.

Figure 6. Dynamic step-scan FT-IR difference spectrum of 10 mM Horse MbCO/D₂O, 25 μm pathlength, 15 μs gate width, 8 cm⁻¹ resolution, laser pump power density 10 mJ/pulse/cm²,

Figure 7. LANL IR difference spectrum of 0.8 mM sperm whale MbCO/D₂O, 50 μm pathlength, effective 4 cm⁻¹ resolution, laser pump power density 3 mJ/pulse/cm², and laser beam diameter 0.5 mm.

Figure 8. UV-vis spectrum of 10 mM deoxyMb/D₂O, 25 μ m pathlength. Bands appear at 560 nm (0.136 A.U., visible band) and 435 nm (1.32 A.U., Soret band).

Figure 9. Dynamic step-scan FT-IR difference spectrum of deoxyMb/D₂O, 25 μ m pathlength, 15 μ s gate width, 100 μ s delay time, laser pump power density 10 mJ/pulse/ cm^2 , laser beam diameter 1 cm.

Figure 10. Duke static FT-IR difference spectrum (deoxyMb-MbCO), each sample 10 mM and 25 μm pathlength.

Figure 11. LANL static FT-IR difference spectrum (deoxyMb-MbCO) each sample 0.8 mM and 50 μm pathlength.

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